JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2000, p. 755–762 Vol. 38, No. 2
0095-1137/00/$04.00+0

Molecular Epidemiology of Rabies Virus Isolates from Israel and Other Middle- and Near-Eastern Countries

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Received 16 June 1999/Returned for modification 14 October 1999/Accepted 17 November 1999

A total of 226 isolates of rabies virus from different areas of Israel, including three human isolates and one sample from South Lebanon were identified between 1993 and 1998 by direct immunofluorescence using monoclonal antibodies to the viral nucleoprotein (N). An epidemiological survey based on nucleotide sequence analysis of 328 bp from the C terminus of the N coding region and the noncoding region between the nucleoprotein and the phosphoprotein (NS gene) was performed. Phylogenetic analysis of the isolates from Israel showed that they were related geographically, but not according to host species. Five variants, related groups distributed among four geographical regions, were identified. In each region, rabies virus was isolated from more than one animal species. A comparison of the sequence analysis of rabies virus samples from the rest of world revealed a 2-nucleotide change that distinguished the Middle East variants from the rest.

Between 1949 and 1961, dogs and cattle were the domestic animals most commonly affected by the rabies virus and the jackal was so among wild animals (9). Vaccination programs, mandatory in Israel since 1956, stopped the domestic dog from being a reservoir for rabies virus but did not eliminate rabies from the country. The jackal (*Canis aureus*) was the first wildlife rabies virus vector to be recognized, but extermination of the jackal population in the 1960s reduced its importance as a virus reservoir. The reservoir for rabies virus transmission now persists among wild canids, with occasional transmission to human and domestic animals. Since 1979, the fox (*Vulpes vulpes*) has been the most important reservoir for rabies virus in Israel, and it was recently shown that between 1976 and 1997 foxes accounted for 46% of rabies virus cases whereas only 4% of cases could be traced to jackals (17). Rabies in Israel is enzootic, and cumulative data from 50 years (1948 to 1997) on its geographical distribution, analyzed by decade, showed that different districts predominated at each interval, while no single district predominated in the total number of cases. With the development of an oral rabies virus vaccine for wildlife, control efforts can now be focused on elimination of wild species serving as reservoirs. Nevertheless, the success of control programs can be predicted according to accurate identification of the species serving as a maintenance reservoir within the particular region targeted in a vaccination campaign and accurate determination of the patterns of disease transmission within and between different regions, which might suggest natural barriers to animal movement that can also be exploited in a control program. Traditionally, assumptions about reservoir maintenance were based solely on case surveillance data, which are often subject to submission bias. Genetic analysis of rabies virus isolates can circumvent this bias (e.g., more cases are reported in zones of high human populations and cases are more frequently reported among wild species with commensal habits). Patterns of virus evolution, as evidenced by changes in nucleotide sequences, reflect independent pathways of virus transmission indicative of virus population isolated by geography or animal host. In this paper we analyze rabies virus isolates collected from different regions of Israel. Patterns of virus evolution are used to infer a molecular epidemiology that supports the fox as the principal reservoir host for virus transmission and identifies potential geographic barriers that separate fox populations and interrupt disease transmission.

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MATERIALS AND METHODS

Virus isolates. Three human brains and 223 animal brains, including one from South Lebanon, were submitted to the Pathology Division, Kimron Veterinary Institute. All the samples were tested for rabies virus by direct immunofluorescence antibody (dIFA) test (Centocor, Malvern, Pa.) as described previously (10). The species of origin of the samples from Israel and their geographic origin are shown in Tables 1 and 2.

RNA extraction and RT-PCR. Total RNA was extracted, using TRI reagent (Molecular Research Center, Cincinnati, Ohio), from infected brain tissue for PCR assay according to the manufacturer's instructions. For reverse transcription (RT), RNA was heated to 95°C for 1 min, cooled on ice, and added to 20 μl of a reverse transcription reaction mixture containing avian myeloblastosis virus (AMV) RT reaction buffer (25 mM Tris-HCl [pH of 8.3 at 42°C], 25 mM KCl, 5 mM $MgCl_2$, 5 mM ditheothreitol, 0.25 mM spermidine), 250 μM concentrations of each of four deoxynucleotides, 100 pmol of specific primer 10 g (5′-CT ACAATGGATGCCGAC-′3) (11), 25 U of RNasin (Promega, Madison, Wis.), and 10 U of AMV reverse transcriptase (Promega). After incubation at 42°C for 90 min, 1 μl of cDNA product was added to a 25-μl (total volume) PCR mixture [60 mM Tris HCl, 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$ (pH 8.5)] containing 100 μM concentrations of each of the four nucleotides, *Taq* polymerase (5 U of Amplitaq; Perkin-Elmer), and 100 ng each of primer 113 (5′-GTAGGATGAT ATATGGG-′3) (15) and primer 304 (5′-GAGTCACTCGAATATGTC-′3) for PCR. Forty cycles of 45 s at 94°C, 45 s at 37°C, and 90 s at 72°C were programmed. The PCR product of 521 bp was analyzed on a 1.5% agarose gel containing ethidium bromide.

Genetic and computer analysis. Genetic analysis was performed on PCR products from the brains of humans and wild and domestic animals. Analysis of the nucleic acid sequences was carried out with the Pileup program, a part of the Genetics Computer Group Wisconsin sequence analysis package (3). The 521-bp PCR products of the N genes were purified (Wizard PCR prep DNA purification system; Promega) and sequenced with an Applied Biosystems automatic sequencer and one of the PCR primers. For the 521-bp sequence we used NS primer 304 (genome position, nucleotides [nt] 1513 to 1533), which was selected according to the Pasteur virus sequence (16). The 328-bp sequences of the N carboxy-terminal nt 1156 to 1484 were compared. This fragment contained 264 bp of the N gene and 64 bp of the untranslated region between the N and NS genes. Fourteen isolates originating from the Middle East, Europe, and Africa (6) were studied and compared to the sequences of the isolates from Israel.

Nucleotide sequence accession numbers. The nucleotide sequences described in this report have been submitted to GenBank and assigned the following accession numbers: variant I, AF162801 to AF162807; variant II, AF162808 to

TABLE 1. Israeli rabies virus samples grouped according to species and year of isolation

Animal species	No. of samples collected during:				
	1993	1995	1996	1997	1998
Fox	2	9	23	57	40
Dog	1	1	7	8	9
Jackal		1	2	2	3
Cattle		1	15	6	17
Cat			1	1	3
Wolf				6	1
Badger			2	1	
Rabbit				1	
Human			1	2	
Goat					1
Stone marten				1	1
Total	3	12	51	85	75

AF162817; variant III, AF162818 to AF162827; variant IV, AF162828 to AF162830; and variant V, AF162831 and AF162832.

RESULTS

Identification of rabies virus by dIFA and RT-PCR assays. Brain tissues collected from 226 animals and humans dying from rabies from 1993 to 1998 were distributed as follows (Table 2): 3 samples from 1993, 12 samples from 1995, 51 samples from 1996, 85 samples from 1997, and 75 samples from 1998. All samples were rabies virus-positive by dIFA and RT-PCR assays.

Genetic analysis. Overall, the virus samples from Israel shared more than 97% nucleotide homology. According to patterns of nucleotide substitution (Fig. 1 and 2) the 226 virus samples were separated into five groups or genetic virus variants. With the exception of variant V, inclusion of a virus isolate within a group was based solely on its geographic origin (Fig. 3). All 57 isolates originating from the Golan Heights region, regardless of animal species, were grouped as variant I. Variant I revealed seven subgroups, designated A to G (Fig. 1), which were characterized by a change at nt 45 (T→C) (Fig. 2). The 83 isolates originating from Galilee were grouped as variant II. Variant II contained 10 subgroups, 8 of which, A, B, C, D, E, F, I, and J (Fig. 1), were characterized by a change at nt 180 (G→A) (Fig. 2). Subgroup G was identical to the consensus sequence. Subgroup H had a change at nt 146 (A→G) (Fig. 2). The 58 isolates originating from central and southern areas of the country were grouped as variant III, which is characterized by a change at nt 295 in the noncoding region between the N and NS genes from C to T (Fig. 2). The 28 isolates originating from Arava Valley were grouped as variant IV, with three subgroups, A, B, and C (Fig. 1), characterized by changes at nt 248 (A→G) and nt 257 (T→C) (Fig. 2). Group V consisted of only two samples, one from a dog and one from a cow, from the Golan Heights and Arava Valley, respectively; this variant was quite different from all the other variants from Israel and differed in six positions (nt 55, 87, 156, 178, 272, and 281) (Fig. 2). All groups contained samples from at least two different animal species.

Human rabies virus. Three human rabies virus cases were recently diagnosed in Israel within a 13-month period (2). The first patient, a 20-year-old soldier in the Golan Heights, was bitten on his lips on 6 October 1996 by an unidentified animal. On 16 November 1996, he was admitted to the emergency room, and on 15 December 1996, 35 days after clinical symptoms appeared, he died, despite supportive therapy. The second patient, a 7-year-old girl from Kalanswa, a village in central Israel, was admitted, unconscious, to the hospital on 21 November 1997. The only potential exposure to rabies identified in her case history was a wound that had been inflicted 2 months earlier by an unidentified animal that attacked her in her sleep. The child died on 7 December 1997, despite supportive care. The third patient, a 58-year-old man from a northern village, Judieda, was admitted to the emergency room on 11 December 1997 with a sore throat. It transpired that he had been bitten on his left hand and face 3 months earlier, while sleeping. The patient died on 16 December 1997. The rabies virus variants isolated from these cases belonged to three distinct geographical regions: the Golan Heights region, Galilee, and the Central-Southern region (Fig. 3). The patterns of nucleotide substitution of the three human isolates, classified in subgroups I A, II F, and III B, respectively, were identical to those of fox isolates from the same regions (Fig. 1).

Relationship with foreign rabies virus isolates. All the Israeli isolates, the South Lebanon isolate, and the Near-Eastern variants from Oman, Saudi Arabia, and Iran were closely related. Israeli rabies virus variants, except variant V, had 98% homology with the Omani red fox, Saudi Arabian red fox, and Iranian dog and wolf isolates (Fig. 4). Israeli variant V differed from other Israeli variants in sharing 97% homology with both Omani and Saudi Arabian red fox isolates, 96% homology with Iranian wolf isolates, and 97% homology with Iranian dog isolates. Two nucleotide substitutions were detected at nucleotide positions 310 (G) and 313 (T), and these characterized the Middle Eastern isolates (Fig. 5). The Israeli isolates were found to be more closely related to European isolates (93 to 96% homology) than to African isolates, e.g., the Egyptian human isolate (90% homology) (Fig. 4).

DISCUSSION

Molecular epidemiology based on RT-PCR is an important tool for the classification of animal virus diseases, including rabies virus, and provides a better understanding of epidemiological relationships (1, 4). The nucleotide sequence analysis of the 328-bp fragment of the N gene of isolates from Israel represented the first epidemiological study done in Israel. In Israel, rabies is region but not host specific, and isolates are grouped into four geographical regions. Similar geographical distributions were reported in the Canadian province of On-

TABLE 2. Animal species distribution of rabies virus isolates according to geographical region of Israel

Species	No. of isolates from:				Total no. of isolates
	Golan Heights	Galilee	Central-Southern region	Arava Valley	
Fox	26	47	38	20	131
Dog	4	9	12	1	26
Jackal	3	4	1		8
Cattle	21	16	2		39
Cat	1	2	2		5
Wolf				7	7
Badger		1	2		3
Rabbit		1			1
Human	1	1	1		3
Goat		1			1
Stone marten	1	1			2
Total	57	83	58	28	226

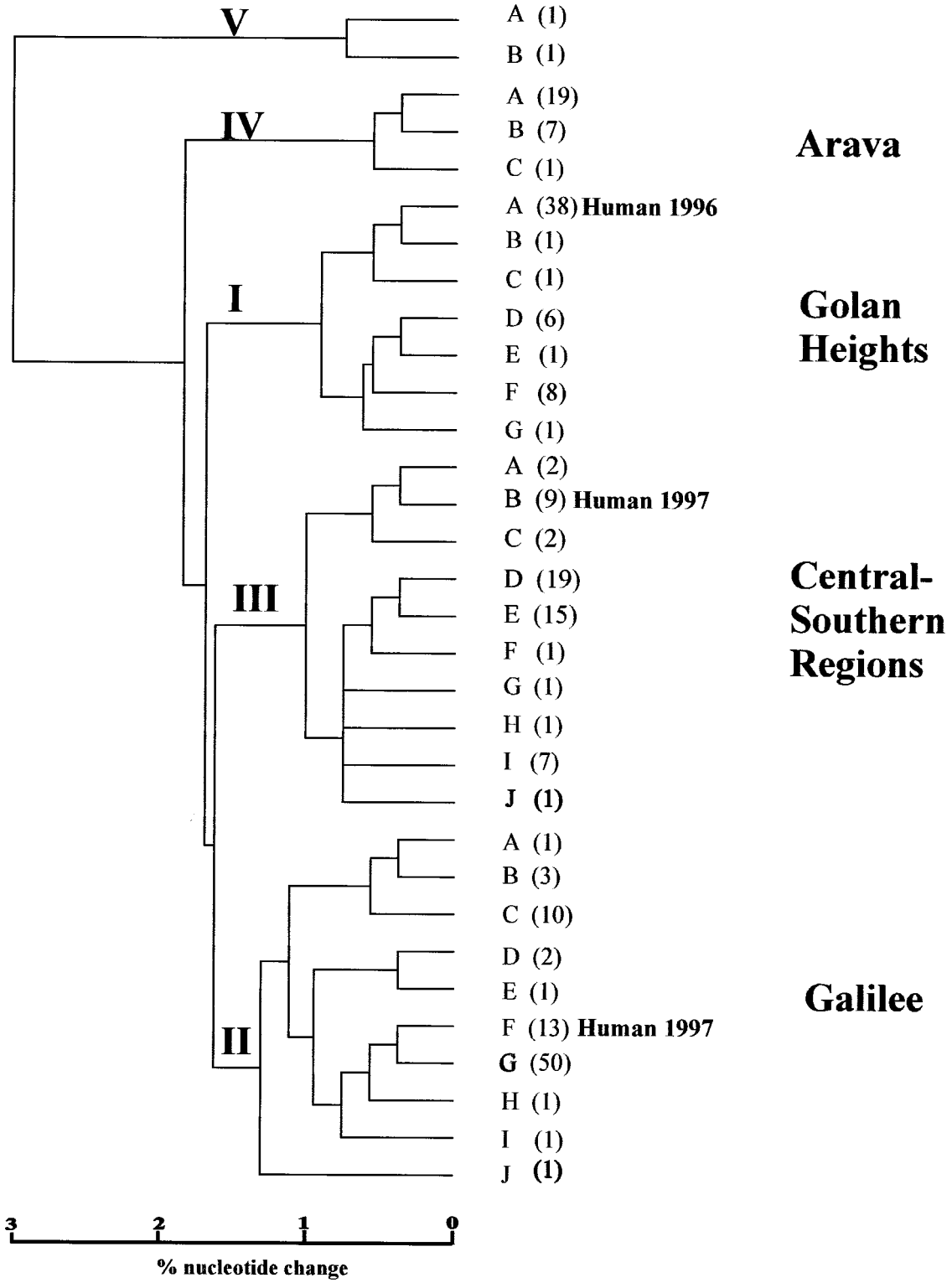


FIG. 1. Dendrogram showing the percentage of genetic relatedness among Israeli rabies virus isolates, as determined by analysis of 328-bp sequence of the nucleoprotein gene. The numbers in parentheses are the numbers of isolates in each subgroup.

tario after PCR and restriction enzyme analysis of the N gene product (8), and similar studies based on analysis of a 320-bp fragment have contributed to the epidemiology of rabies in the United States (12), Venezuela (7), and Africa (13), while the African wild dog isolates from the Masai Mara in Kenya were clustered according to the 304-bp sequence from the N gene (5). Phylogenetic data suitable for compilation of a large epidemiological study were provided by using a shorter sequence, that of a 200-bp region of the N gene, in a study of 87 isolates collected from areas where dog rabies virus is enzootic in Asia,

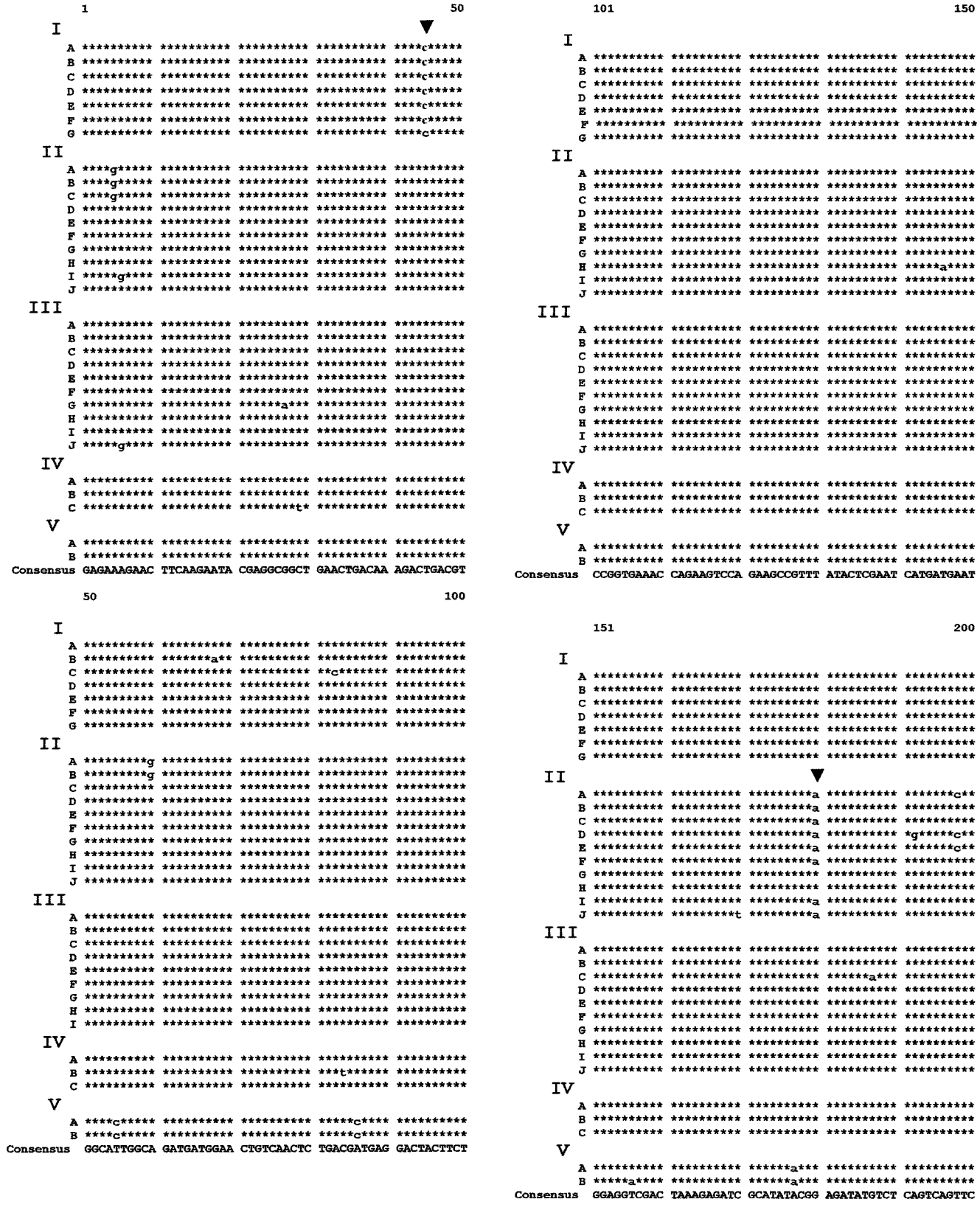
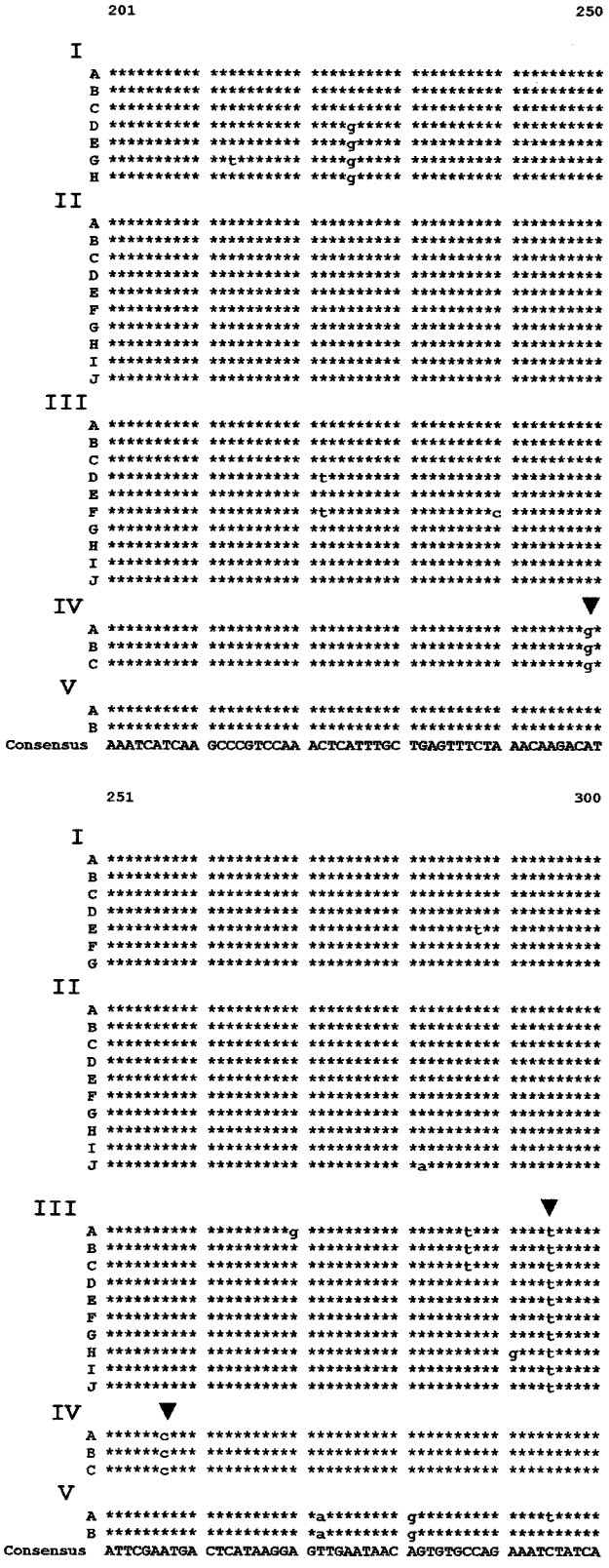


FIG. 2. Nucleotide sequence homology analysis of 226 Israeli rabies virus isolates. Arrowheads indicate the positions of the nucleotide substitutions characteristic of each sequence group.



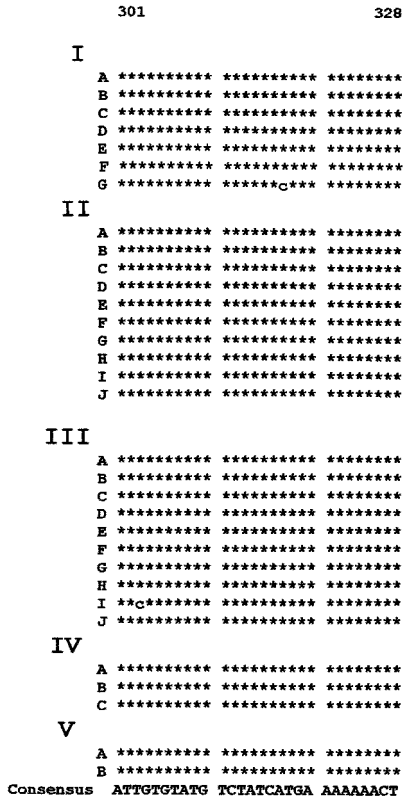


FIG. 2—*Continued.*

Africa, Europe, and the Americas (14). Phylogenetic analysis of the 93-bp noncoding region corresponding to the 3′ end of the N gene, the intergenic N-NS region, and the 5′ end of the NS gene of 69 rabies virus isolates from various parts of the world facilitated their geographical classification (6). Nucleotide sequence analysis of the virus isolates from Israel revealed that differences between the four groups of variants resided in only one or two nucleotides. Since mutations in this region of the viral genome are rare, nucleotide differences between variants can serve as a marker (16). Rabies variant V, of which two isolates were included in the present study, differed from the other variants in six nucleotides. These two variants were isolated from a dog (VI A) and a cow (VI B) in villages situated along the Israeli borders with Jordan and Syria, respectively, and it is most likely that they represent viruses that are prevalent in those countries. An alternative explanation is that the animals moved from the site where the infection occurred to a different area, where their infection status was detected. It was established that foxes serve as a reservoir for rabies virus in Israel, so geographic boundaries such as mountain ranges and bodies of water could effectively serve to isolate animal populations; therefore, geographic parameters might be responsible for the separate virus populations. The borders of each region are based on the genetic data presented in this work together with the geographic features of Israel and the neighboring countries. The Israeli rabies virus isolates fall into four main geographical regions: the Golan Heights (region 1), Galilee (region 2), the Central-Southern area (region 3), and Arava Valley (region 4) (Fig. 3). The Golan Heights region was bound by the Syrian, Jordan, and Lebanese borders on three sides, and on the west side, it was bound by the Jordan River and the Kinerret Lake, which separate regions 1 and 2. The Galilee region (region 2) is bound by the Lebanese border in the north and by the Carmel and Gilboa Mountains in the south. The Central-Southern region (region 3), which is the most populated area, is bound by the Jordan River and the

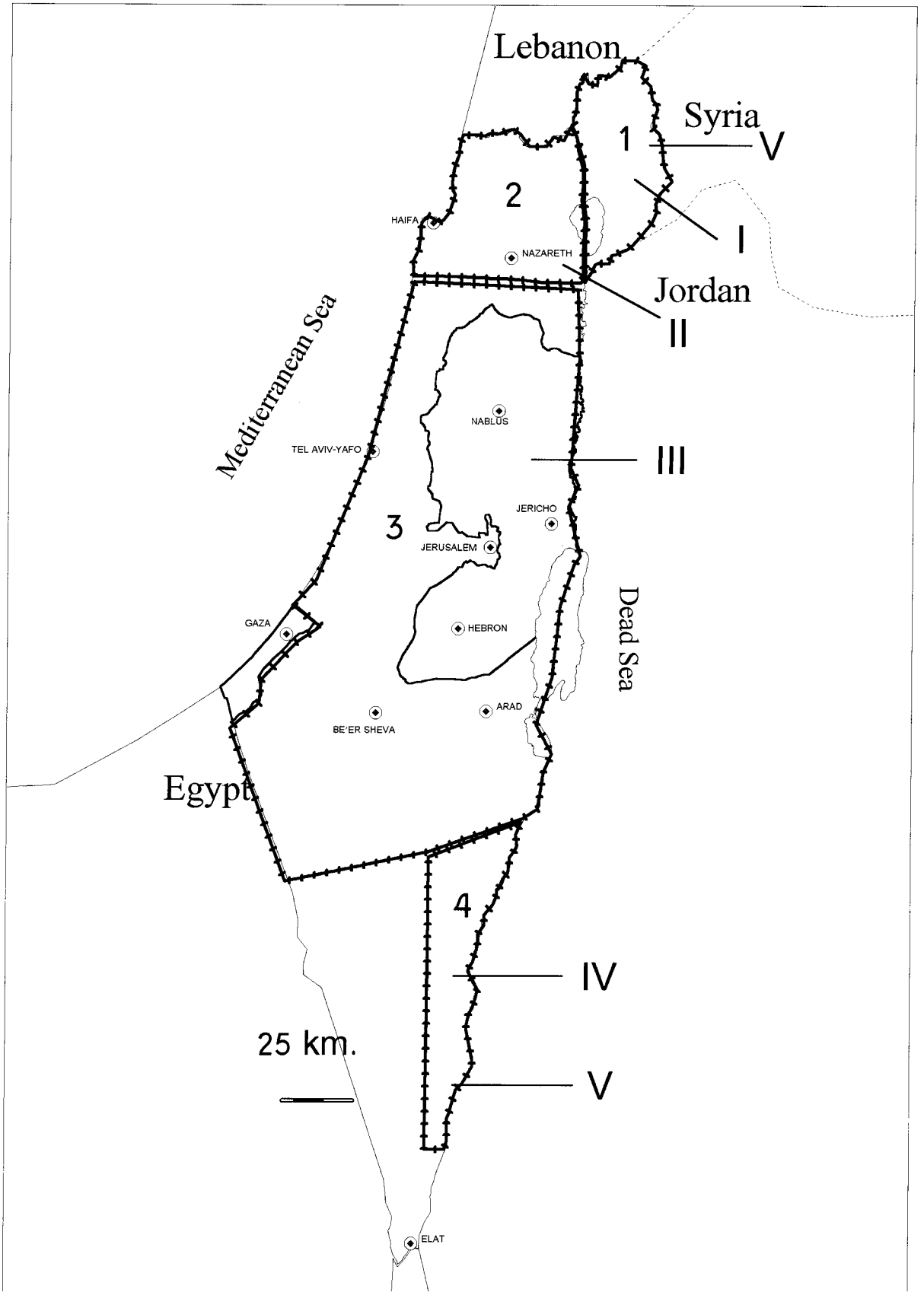


FIG. 3. Map of Israel showing the distribution of the five virus variant groups (I to V) found in four geographical regions designated regions 1 to 4 and defined as follows: region 1, Golan Heights; region 2, Galilee; region 3, Central-Southern area; and region 4, Arava Valley.

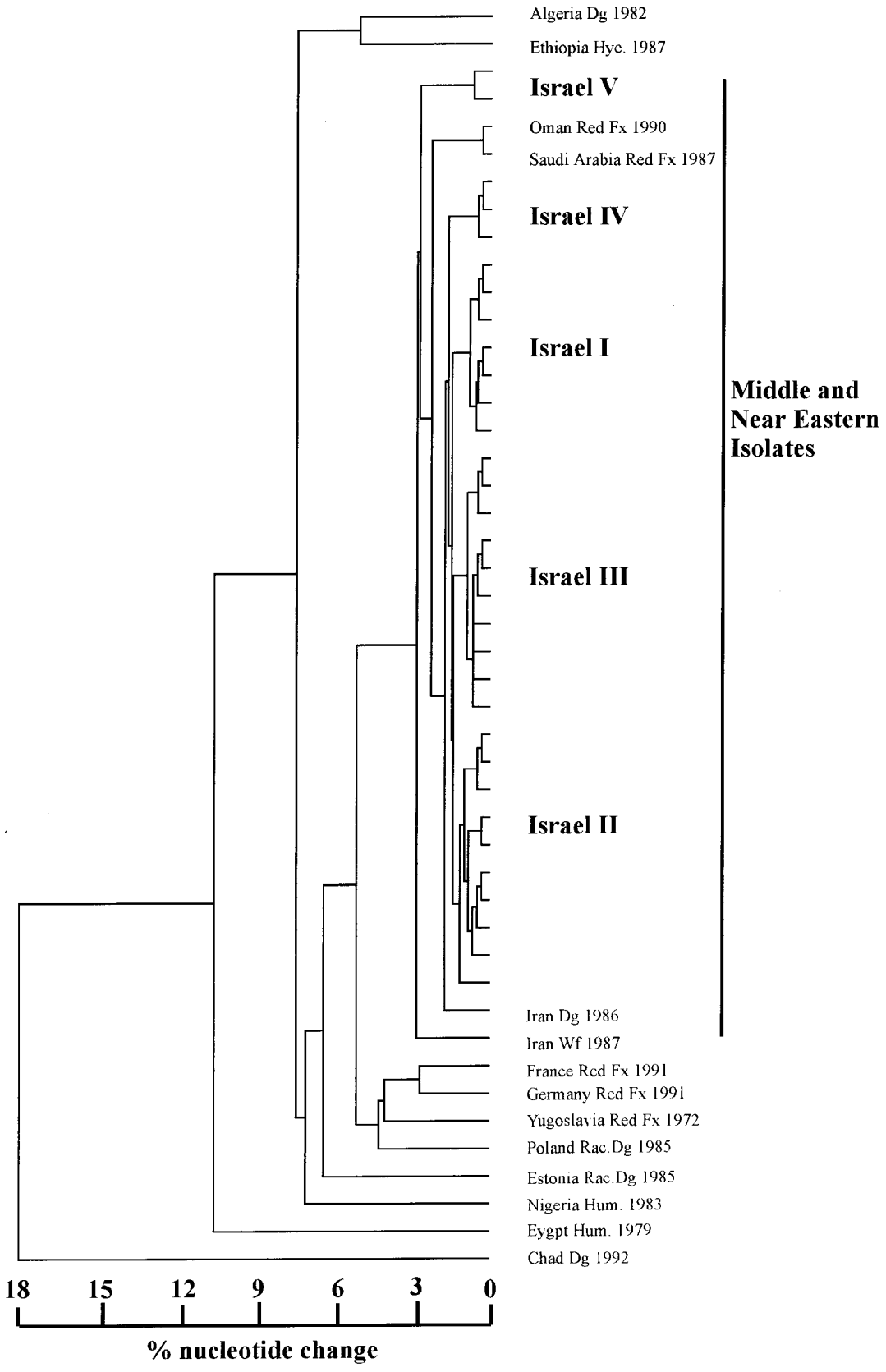


FIG. 4. Phylogenetic tree produced by Pileup program based on 328-bp sequence of foreign isolates and the Israeli variants. The foreign isolates included isolates from the African countries Algeria (U22643), Ethiopia (U22637), Nigeria (U22488), Chad (U22644), and Egypt (U22627); the European countries France (U22474), Germany (U22475), Yugoslavia (U22839), Poland (U22840), and Estonia (U22476); and the Middle-Eastern countries Iran (U22482 and U22483), Oman (U22480), and Saudi Arabia (U22481). Dg, dog; Hye, hyena; Fx, fox; Wf, wolf; Rac., raccoon; Hum., human.

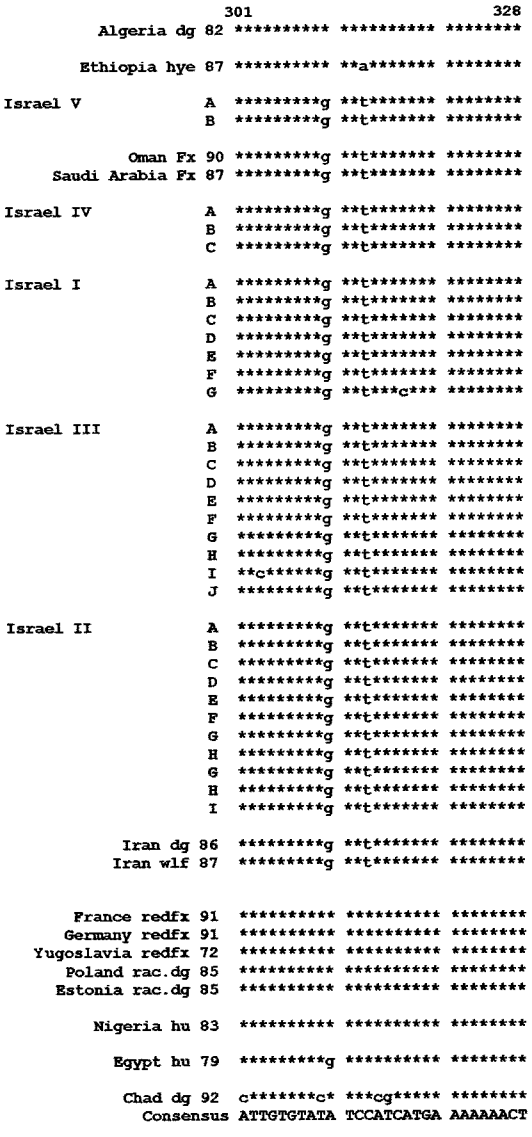


FIG. 5. Sequence analysis of foreign virus isolates and the Israeli variants. dg, dog; hye, hyena; Fx, fox; wlf, wolf; redfx, red fox; rac., raccoon; hu, human.

Negev desert. The Arava Valley region (region 4), which is the least-populated area, is bordered by the Jordan border and the Negev desert. Evidence for the geographical distribution of the Israeli isolates was received from the molecular and antigenic analysis of the three human isolates. The results showed that there are three genetic variants, which differed from each other by one or two nucleotides associated with the three geographic regions. The antigenic characterization of the human isolates revealed two phenotypic variants, 1 and 2, which were distributed in two different geographic regions (2). Phenotype 1 is distributed in northern and southern Israel, and phenotype 2 is located in central Israel. Variants from Israel were very closely related to isolates from the Middle-Eastern countries or regions of South Lebanon, Iran, Oman, and Saudi Arabia (Fig. 4). Recent molecular studies showed differences between isolates from Europe, Asia, the Americas, and Africa (6, 14), while a close relationship was found between isolates from Middle-Eastern and European countries, though they differed from isolates of Asian and African countries (6). The genetically close relationship between isolates from European and Middle-Eastern countries suggests that the viruses circulating in foxes in both these regions might have the same origin; nevertheless, it seems that the 2-nucleotide substitution is a specific genomic marker for rabies virus isolates found in Middle-Eastern countries.

ACKNOWLEDGMENTS

We thank C. E. Rupprecht from the National Center for Infectious Diseases, Atlanta, Ga., for helpful discussions.

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